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Heterologous prime-boost regimen adenovector 35-circumsporozoite protein vaccine/recombinant Bacillus Calmette-Guérin expressing the *Plasmodium falciparum* circumsporozoite induces enhanced long-term memory immunity in BALB/c mice

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ABSTRACT

Background: Sustained antibody levels are a hallmark of immunity against many pathogens, and induction of long-term durable antibody titers is an essential feature of effective vaccines. Heterologous prime-boost approaches with vectors are optimal strategies to improve a broad and prolonged immunogenicity of malaria vaccines.
Results: In this study, we demonstrate that the heterologous prime-boost regimen Ad35-CS/BCG-CS induces stronger immune responses by enhancing type 1 cellular producing-cells with high levels of CSp-specific IFN-γ and cytophilic IgG2a antibodies as compared to a homologous BCG-CS and a heterologous BCG-CS/CSp prime-boost regimen. Moreover, the heterologous prime-boost regimen elicits the highest level of LLPC-mediated immune responses.
Conclusion: The increased IFN-γ-producing cell responses induced by the combination of Ad35-CS/BCG-CS and sustained type 1 antibody profile together with high levels of LLPCs may be essential for the development of long-term protective immunity against liver-stage parasites.

1. Introduction

Although a successful eradication of certain infectious diseases such as smallpox has been realized, vaccination strategies against human pathogenic parasites remain a fundamental challenge for biomedical research [1]. Long-lasting protective antibody production is one of the hallmarks of effective vaccination and is an important feature of immunological memory [2].

The clinically silent liver stage of *Plasmodium* infection epitomizes an attractive target for antimalarial vaccine development [3,4]. However, despite decade long endeavors, no antimalarial vaccines have been licensed today. Nevertheless, promising results are emerging despite the fact that the leading pre-erythrocytic subunit vaccine candidate (RTS,S) has proven to be only partially protective in clinical trials [5].

In the previous study, we have shown that a recombinant (r) BCG expressing the *Plasmodium falciparum* circumsporozoite protein (BCG-CS) induced activation and priming of CSp-specific immunity in BALB/c mice [6]. A prime-boost regimen consisting of this BCG-CS combined with adenovector 35 (Ad35) expressing the same antigen (Ad35-CS) is utilized in this work. Based on evidences in literature we conclude that a reasonable strategy to induce broad and prolonged immune response against malaria infection may be realized by priming with recombinant virus and boosting with rBCG [7–9]. Therefore, a rBCG provides an option that can fit within the existing World Health Organization (WHO) expanded program of immunization (EPI) considering that BCG is being given at birth.

Since a major concern is, how to induce protective cell-mediated immunity (CMI) particularly IFN-γ-producing $CD8^+$ T cells, which have been shown to provide long-term immunity to malaria [10]. These cells are essential in combating parasitic infections, including malaria. Due to intracellular expression of the CSp insert in the rAd35 genome and the intracellular residence of BCG expressing

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the same antigen, we propose that BCG-CS is likely an efficient route of antigen delivery. Moreover, the expressed CSp is expected to be processed in the cytosolic pathway and presented efficiently with class I major histocompatibility (MHC) molecules [11,12].

During parasitic infection, the immune response mediated by $CD4^+$ and $CD8^+$ T cells is crucial for effective protection, also against malaria [13]. The induction of antigen-specific long-lived immune responses accompanied by an expansion of $CD4^+$ and $CD8^+$ T cells plays a pivotal role in malaria vaccine development. To accomplish this, it is therefore important to investigate optimal prime-boost strategies.

Sustained antibody levels are a hallmark of immunity against many pathogens, and induction of long-term durable antibody titers is an essential feature of effective vaccines. In the context of humoral immunity, the ability of a vaccine to confer this long-term immunity depends on both memory B cells and long-lived plasma cells (LLPCs) [14]. Numerous mechanisms have been proposed whereby persistent antibody production can be maintained, such as low-grade chronic infection, repeated antigenic exposure, antigen–antibody complexes, idiotypic networks and cross-reactivity to self or environmental antigens [2]. However, more recent investigations have shown that antibody titers can persist despite the lack of antigen exposure, for decades. In addition, sustained antibody titers after immunization in humans do not appear to require memory B-cell activation [15]. The source of this long-term antigen-specific antibody has been identified as bone marrow (BM)-resident nonproliferating plasma cell subsets called LLPCs [16,17]. We hypothesize therefore that the long-term response conferred against *P. falciparum* CSp in the present study is due to the capacity of the heterologous prime-boost, Ad35-CS/BCG-CS, to generate markedly enhanced LLPC responses. To this end, we evaluated the quantity and quality of cellular immune responses induced by a heterologous prime-boost regimen using Ad35-CS followed by BCG-CS to induce CSp-specific memory immunity.

In this study, we demonstrate that the heterologous prime-boost regimen Ad35-CS/BCG-CS induces stronger immune responses by enhancing a type 1 cellular immune response with high levels of CSp-specific IFN-γ producing-cells and cytophilic IgG2a antibodies as compared to the homologous BCG-CS and the heterologous prime-boost BCG-CS/CSp regimen. Moreover, we show that the heterologous prime-boost regimen elicits the highest level of LLPC-mediated immune responses.

2. Materials and methods

2.1. Animals and immunization procedures

The immunization procedures were performed according to the Swedish Animal Act and were approved by the Swedish Animal Care and Ethical Review committee. Six to eight week-old female BALB/c mice were obtained from NOVA-SCB (Sollentuna, Sweden) and were housed in specific pathogen-free conditions in the animal facility at Stockholm University. The BCG-CS was formulated in PBS with 0.05% Tween 80 and administered subcutaneously (s.c.) at the dorsal neck at a dose of 10^6 colony forming units (CFU) in a total volume of 100 μl. The Ad35-CS was formulated in PBS and was given intramuscularly (i.m.) at the gastrocnemius muscle at a dose of 10^9 viral particles (vp) in a total volume of 50 μl (*i.e.*, 25 μl in each leg). Boosting immunizations were given 4-week post-priming in the same procedure as above in all cases.

2.2. Recombinant BCG-CS and AD35-CS vaccine constructs

The BCG-CS and Ad35-CS constructs, expressing CSp, have been described previously [6,18].

Table 1
Standard immunization regimes.

BALB/c mice	Prime week 0	Boost week 4
Group 1 (8 mice)	BCG-CS, 10^6 CFU	BCG-CS, 10^6 CFU
Group 2 (8 mice)	BCG-CS, 10^6 CFU	CSp, 5 μg
Group 3 (8 mice)	Ad35-CS, 10^9 vp	BCG-CS, 10^6 CFU
Group 4 (4 mice)	Ad35-CS, 10^9 vp	Ad35-CS, 10^9 vp

2.3. Immunization design and dosage

The immunization design and the dosage of the different vaccines are summarized in Table 1.

2.4. Peptides and proteins

Specific responses to *P. falciparum* CSp were measured by stimulating splenocytes and LLPCs with peptides deduced from the CSp antigen; namely, the C-terminal (C-CSp, PfCS282-383), N-terminal (N-CSp, PfCS22-110) and immunodominant $CD8^+$ T cell epitope (IDE-CSp, PfCS-NYDNAGTNL). The synthesis and immunological characterizations of those peptides have been reported in details elsewhere [19,20]. The rCSp was provided by Crucell (Leiden, The Netherlands) and has been described elsewhere [12].

2.5. Isolation of splenocytes and long-lived plasma cells

Spleen-cell suspensions were prepared by teasing the organ with sterile forceps followed by passing through 27G needles several times, and then centrifugation. Bone marrow (BM) cells were collected from the BM of femurs and tibias by flushing them with RPMI. Red blood cells (RBC) were removed by resuspending cells in ACK RBC-lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA in dH_2O and adjusted pH to 7.2–7.4 with 1 M HCl; all compounds were purchased from Sigma–Aldrich, Steinheim, Germany) for 5 min before adding excess of RPMI. Splenocytes and LLPCs were purified by centrifugation and resuspended in complete RPMI (RPMI 1640, 10% FCS, 100 IU/ml penicillin, 100 mg/ml streptomycin, 4 mM L-glutamine).

2.6. CSp-specific antibody detection in the serum of immunized mice

CS-specific antibody responses were assessed by ELISA. Ninety-six-well microtiter plates (Costar 96-well HB half Area plate, Corning Inc, NY) were coated overnight with 2 μg/ml CSp in 0.05 M carbonate buffer (pH 9.6) at room temperature. Plates were washed three times with PBS/0.05% Tween 20 and a 1:400-dilution of individual serum samples were added to corresponding wells and a serial dilution of 2-fold with PBS/0.05% Tween 20. Plates were incubated for 2 h at room temperature and were washed three times and incubated with alkaline phosphatase-labeled anti-mouse IgG (Southern Biotech, Birmingham, AL, USA). For detection of IgG subclasses, samples were incubated with alkaline phosphatase-labeled anti-mouse IgG1 or IgG2a antibodies (Southern Biotech, Birmingham, AL). The enzyme/substrate reaction was developed using p-nitrophenyl phosphate (Sigma–Aldrich, Steinheim, Germany). Optical density was measured at 405 nm by using a V max ELISA reader (Molecular Devices Instruments).

2.7. CSp-specific T-cell responses in splenocytes of immunized mice

CSp-specific cellular immune responses in vaccinated mice were measured using an IFN-γ ELISPOT assay. The splenocytes from each group of mice were stimulated with a pool of

P. falciparum CSp peptides consisting of C-CSp (PfCS282-383), N-CSp (PfCS22-110) and IDE-CSp (PfCS-NYDNAGTNL). Multiscreen plates (96-wells) were pre-coated with anti-mouse IFN-γ (Mabtech, Nacka, Sweden) and blocked with complete medium (RPMI 1640 containing 10% FCS supplemented with 1 mM L-glutamine and penicillin–streptomycin) for 30 min. After removing the medium, splenocytes from individual mice at a density of 10^5 cells/well were stimulated with a pool of CSp peptides at a concentration of 5 μg/well for 48 h at 37 °C 5% CO_2. Following incubation, plates were washed five times with PBS and were then incubated with 1 μg/ml of biotinylated anti-mouse antibodies (Mabtech) in PBS containing 0.5% FCS for 2 h at room temperature. After washing five times with PBS to remove free biotinylated anti-mouse antibodies, plates were incubated for 2 h with detection antibodies conjugated to streptavidin–alkaline phosphatase at 1:1000 dilutions in the same buffer as above. The enzyme reaction was developed with nitroblue tetrazolium bromo-4-chloro-3-indolyl-phosphate chromogen substrate (Mabtech). The spot-forming units (SFU) per 10^5 cells were counted using a dissection microscope (Carl Zeiss, Stemi 2000-C).

2.8. Detection of CSp-specific LLPCs in BM of immunized mice

Multiscreen HTS-IP Filter Plates (96-wells, Millipore) were pre-wetted with 70% ethanol for 2 min, washed five times with PBS and coated with 5 μg/ml of CSp in PBS overnight at 4 °C. Plates were blocked for 2 h at room temperature with complete medium. BM cells (10^5 cells per well) from the immunized mice were seeded in duplicates and stimulated individually with the C-CSp, N-CSp or IDE-CSp. Plates were incubated for 12 h at 37 °C, 5% CO_2 and 85% humidity. After the incubation period plates were washed five times with PBS and incubated for 2 h at room temperature with HRP-conjugated goat anti-mouse IgG (1:1000; Southern Biotech) in PBS, 5% FCS. After washing with PBS five times, the reaction was developed using a Vectastain 3-amino-9-ethylcarbazole (AEC) substrate kit (Vector laboratories, Burlingame, CA) according to manufacturer's instructions. The reactions were stopped by washing plates with deionized water. Plates were dried in the dark and spots were counted using a dissection microscope (Carl Zeiss, Stemi 2000-C).

2.9. Statistical analysis

Data were analyzed using GraphPad Prism Version 5 (Graphpad Software, Inc., San Diego, CA). The nonparametric Kruskal–Wallis test was used for the comparison of means in different groups. For all tests, $p \leq 0.05$ was considered significant.

3. Results

3.1. Immunogenicity of a heterologous prime-boost regimen

The combination of Ad35-CS and BCG-CS in a heterologous prime-boost regimen resulted in high-levels of CSp-specific IgG responses (Fig. 1). Moreover, antibody responses exhibited higher IgG2a (Th1-type responses) when comparing heterologous prime-boost Ad35-CS/BCG-CS to homologous prime-boost BCG-CS/BCG-CS immunizations (Fig. 1). Among the three CSp peptides tested (C-CSp, N-CSp and CSp-IDE), the response to C-CSp was synergistic and induced stronger IgG2a response in the group primed with Ad35-CS and boosted with BCG-CS (Fig. 2). We next investigated whether the Ad35-CS/BCG-CS prime-boost regimen results in a Th1 immune response, which is predominantly effective in defense against intracellular infection (virus and parasite infections). A group of mice were primed with BCG-CS and boosted with CSp (heterologous prime-boost BCG-CS/CSp). Another group

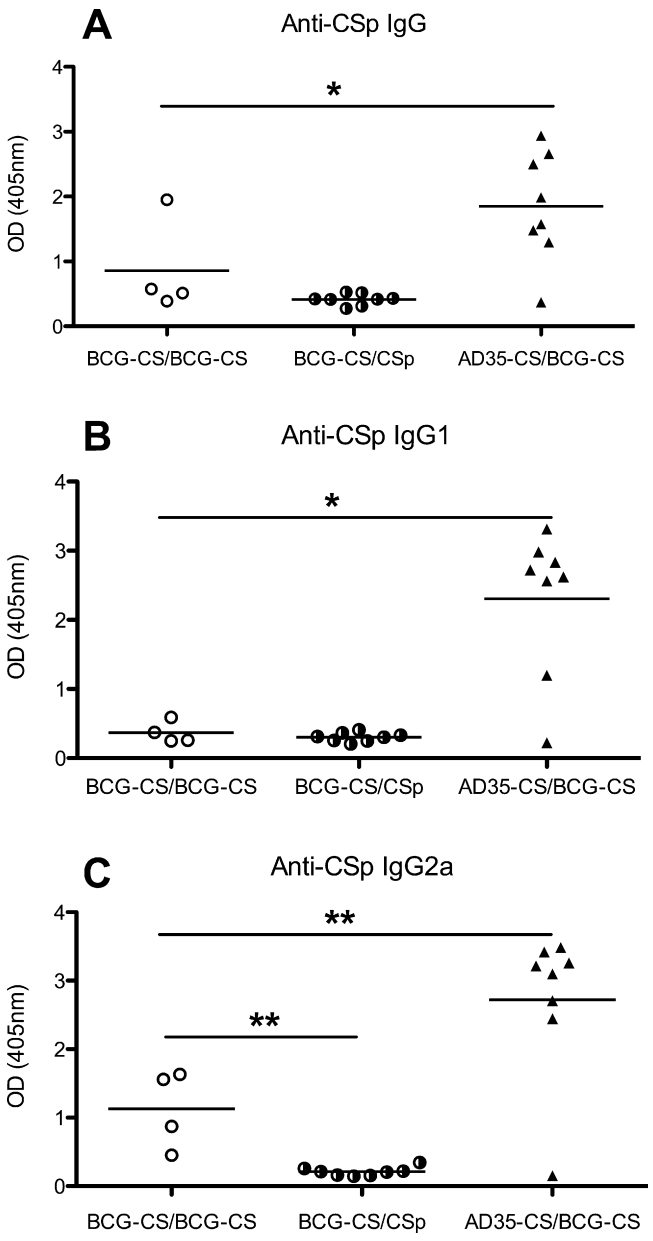

Fig. 1. Effect of heterologous prime-boost Ad-35-CS/BCG-CS regimen on antibody responses against CSp. Immunogenicity of heterologous prime-boost regimen, comprising BCG-CS and Ad35-CS, was analyzed. BALB/c mice (4 per group) were immunized as indicated in the graphs. Two weeks after booster immunization, CSp-specific humoral immune responses were assessed by evaluating CSp-specific IgG (A), IgG1 (B) and IgG2a responses (C) using ELISA. Circle and triangle legends show results from each individual mouse and the horizontal bars represent mean OD values of two independent experiments for each group. Statistically significant differences are shown by asterisks ($^*p < 0.05$, $^{**}p < 0.01$).

of mice were primed with Ad35-CS and boosted with BCG-CS (heterologous prime-boost Ad35-CS/BCG-CS). A control group of mice received priming immunization with BCG-CS, followed by BCG-CS boosting (homologous prime-boost BCG-CS/BCG-CS). Two weeks after the final boost immunization, mice receiving the heterologous prime-boost regimen, Ad35-CS/BCG-CS, showed significantly higher levels of IFN-γ responses upon re-stimulation with the pool of CSp peptides than mice receiving the BCG-CS/CSp prime-boost regimen (p value <0.05; Fig. 3A), and also a higher response than the control group (Fig. 3A). The numbers of CSp-specific IFN-γ-producing cells, as measured by Elispot assays, were significantly higher in the group of mice that had received the heterologous

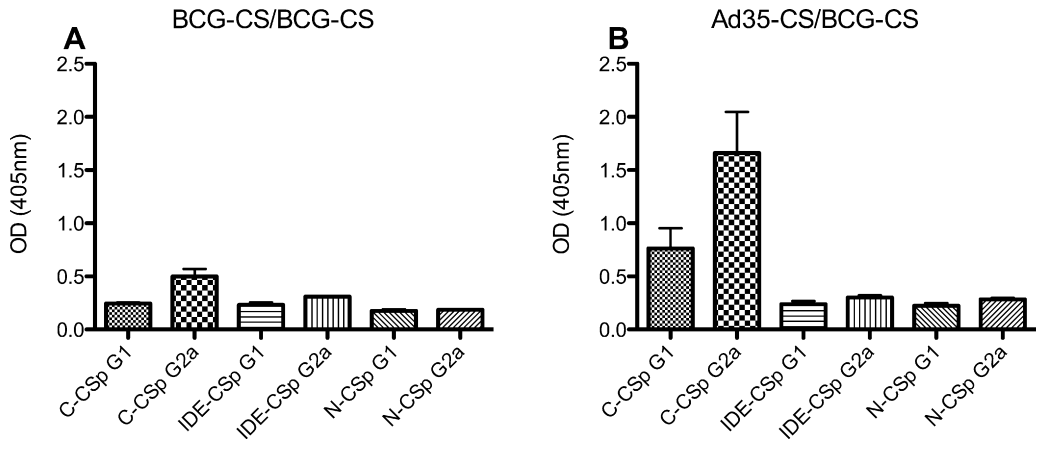

Fig. 2. Effect of heterologous prime-boost Ad-35-CS/BCG-CS regimen on antibody responses against CSp peptides. Immunogenicity of heterologous prime-boost regimen, comprising BCG-CS and Ad35-CS, was analyzed. BALB/c mice (4 per group) were immunized as indicated in the graphs. Two weeks after booster immunization, C-CSp-, N-CSp- and IDE-CSp-specific humoral immune responses were assessed by evaluating IgG1 and IgG2a responses using ELISA. (a and b) Shows (bars) mean OD values of two independent experiments for BCG-CS/BCG-CS and Ad35-CS/BCG-CS groups respectively.

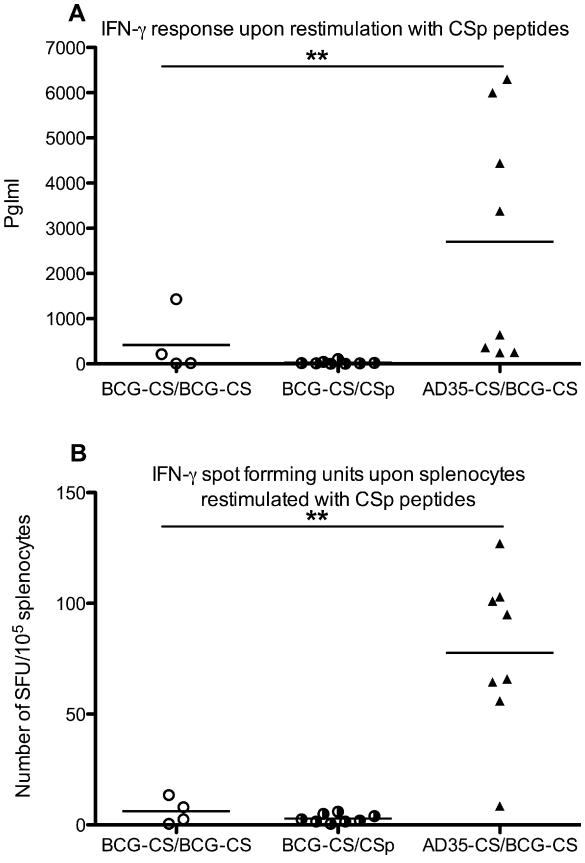

Fig. 3. CSp-specific IFN-γ production by splenocytes. Splenocytes isolated 2 weeks after the last immunization were cultured individually and re-stimulated with a pool of CSp peptides: C-CSp, N-CSp and IDE-CSp. IFN-γ responses were measured after 72 h of stimulation. The individual mouse means of triplicates are presented in circles and triangles and horizontal bar represents group mean value (pg/ml) (A). CSp-specific immune responses of splenocytes were assessed by IFN-γ ELISPOT assay (B). Bars represent means of spot-forming units (SFU) and circles and triangles show SFUs for each individual mouse. Significant differences are shown by asterisks ($^{*}p < 0.05$, $^{**}p < 0.01$). Data represent two independent experiments.

prime-boost regimen Ad35-CS/BCG-CS (p value <0.05; Fig. 3B) compared to the control group.

3.2. Heterologous prime-boost regimen induces antibody-secreting long-lived plasma cells responses

To investigate whether heterologous prime-boosting enhances CSp-specific responses, LLPCs were isolated from BM and stimulated for 48 h with three different peptides generated from the *P. falciparum* CSp, namely C-CSp, N-CSp and CSp-IDE. The ability of LLPCs to secret IgG upon stimulation with the peptides was evaluated by counting spots in ELISPOT. The results are presented as CSp-specific IgG-secreting LLPCs per 10^6 BM cells (Fig. 4A–C). We found that the heterologous prime-boost Ad35Ad35-CS/BCG-CS induced the highest number of CSp-specific IgG-secreting LLPCs. Among the peptides, the LLPC responses to the C-terminus peptide resulted in the highest spot density (Fig. 4A). These results suggest the higher boosting effect of BCG-CS as compared to Ad35-CS, and emphasize the importance of proper priming.

4. Discussion

CSp-based vaccines are yet to be proven sufficiently efficacious for the implementation into human vaccination practice. Efforts to identify strategies of enhancing immune responses of CSp-based vaccination have received a lot of interest and various delivery systems have been emerging. The key strength of this concept is that a greater level of immunity is established by heterologous prime-boost than can be attained by a single vaccine administration or homologous boost strategies [21,22]. In this work, we explored the impact of heterologous prime-boost of a *P. falciparum* CSp-based vaccine using two different live recombinant vectors systems, rBCG and Ad35. Such approaches are identified as heterologous prime-boost strategies referring to the utilization of different vaccines for priming and boosting to improve the immunogenicity of vaccines. Enhancing the immunogenicity of CSp, the leading malaria preerythrocytic vaccine candidate, will be a very important cornerstone toward controlling or eradicating malaria. Although $CD8^+$ T effector memory cells are required for protection against liver-stage malaria [23], prolonged survival of a subset of plasma cells (PCs) in BM has been implicated as a key component of long-term humoral immunity [2,24]. However, the intrinsic characteristics of the PC subsets, the basis of their longevity, and their actual contribution to durable antibody titers are incompletely

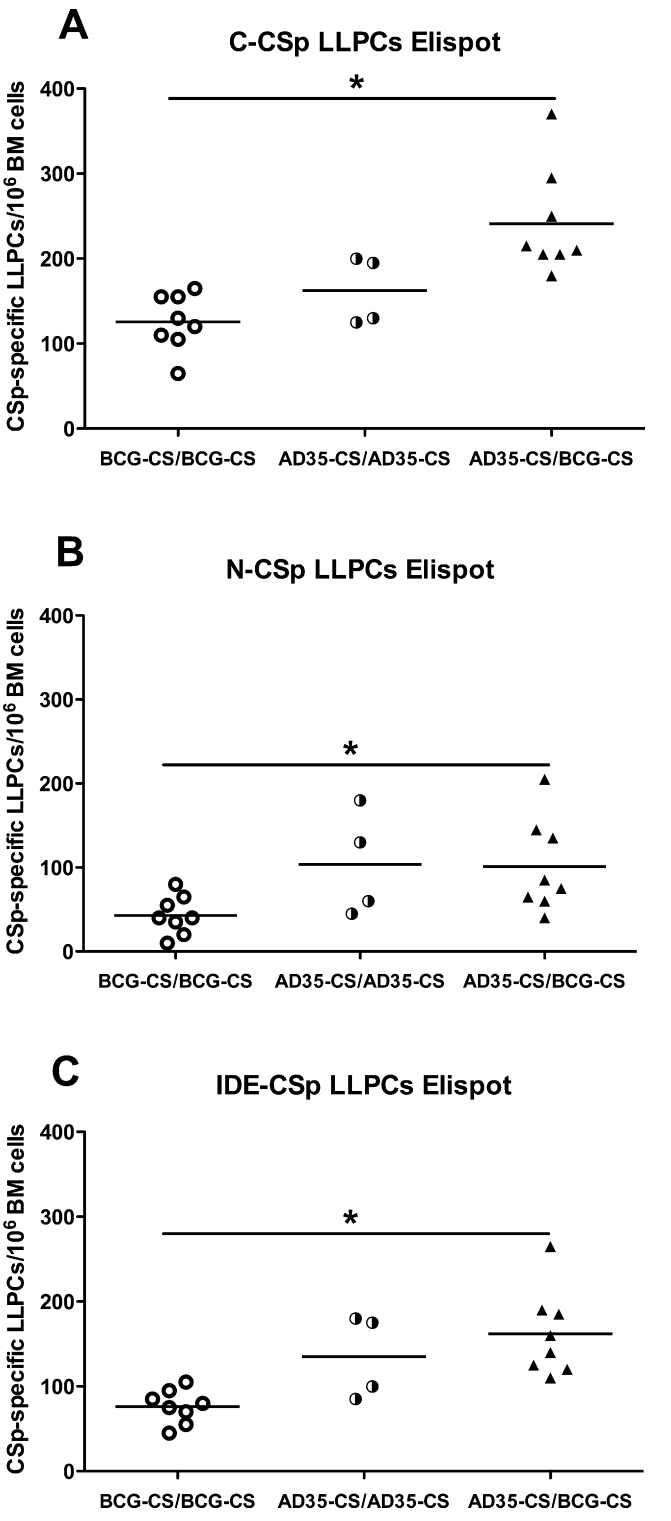

Fig. 4. Effect of heterologous prime-boost vaccination comprised of the BCG-CS and Ad35-CS on CSp-specific antibody-secreting long-lived plasma cells. The number of antigen-specific LLPCs present in BM was enumerated 4 weeks after last immunization of the mice. Specific antibodies to C-CSp- (A), N-CSp- (B), and IDE-CSp- (C) secreting LLPCs are shown for each individual mouse (circles and triangles) as well as group mean value (horizontal bar). Asterisks denote statistical significance ($^{*}p < 0.05$, $^{**}p < 0.01$). Data are representing two independent experiments.

understood. In this study, we employed two approaches (*i.e.*, use of two delivery systems in heterologous prime-boost administration) to enhance the immunogenicity of CSp in BALB/c mice and evaluated the outcome. We have demonstrated that sequential immunization with different delivery systems, the so-called heterologous prime-boost regimen Ad35-CS/BCG-CS, induced significantly stronger immune responses as compared to the homologous immunization. This strategy induced in BALB/c mice a type 1 cellular immune response with high levels of CSp-specific IFN-γ-producing cells and cytophilic IgG2a antibodies as well as induced the highest numbers of LLPCs.

Major obstacles in the development of a vaccination regimen against malaria have traditionally been the lack of immunogenicity of the identified candidate antigens and formulations. It has been suggested that protection in RTS,S-vaccinated children increases when antibody titers against CSp are above the threshold of 18–40 EU/mL. However, RTS,S/AS01E and other RTS,S formulations are still capable of inducing those titers in all vaccinated children despite being partially protective [25]. One way to improve the immunogenicity of antigen is to use different recombinant vaccine platforms such as vectors for antigen delivery [3,26]. Recombinant adenovectors and rBCG are invaluable option among the different vectors since it has been shown that they exhibit efficient adjuvant effects, to enhance immunogenicity and to induce potent memory T- and B-cell responses [27,28]. Interestingly, priming with Ad35-CS and boosting with BCG-CS yielded not only profound CMI but also potent humoral immunity mediated by murine IgG2a cytophilic antibodies, suggesting that this combination might be efficient in inducing protective immunity. This result corroborates previous studies showing that priming with Ad35-CS vaccine followed by RTS,S/AS01B boosting significantly improves immunogenicity to *P. falciparum* CSp [29]. Furthermore, the effect of adenoviral priming was consistent in the other mouse strains and with other antigens such as the *P. falciparum* merozoite surface protein (MSP)–1 [30]. A recent finding from human clinical trial has shown that priming with the recombinant simian adenovirus ChAd63 encoding the preerythrocytic insert multiple epitope thrombospondin-related adhesion protein (ME-TRAP;) and giving a booster immunization 8 weeks later with a modified vaccinia virus Ankara (MVA) ME-TRAP induced high levels of TRAP antigen-specific $CD8^+$ and $CD4^+$ T cells [31].

IFN-γ plays a central mediator role in cellular responses and has been show to be crucial against intracellular protozoan parasites such as *Plasmodium* [32,33]. It is noteworthy to mention that IFN-γ responses to both liver- and blood-stage antigens have been positively correlated with protection [34]. In the same line, we found that the heterologous prime-boost Ad35-CS/BCG-CS induced significantly higher numbers of CSp-specific IFN-γ-producing cells, indicating the induction of a type 1 T-cell response.

The heterologous prime-boost administration also elicited the highest levels of CSp-specific IgG and in particular IgG2a. This finding has great implication for CSp-specific antibody responses, which might confer protection because the IgG response in the current heterologous prime-boost administration was mainly induced against the C-terminal region of CSp domain. The fact that the antibody response was stronger against C-CSp implies that epitopes responsible CSp-specific antibody responses are located in the C-terminal domains of the protein. Prolonged survival of a subset of PCs in BM has been implicated as the key component of the long-term maintenance of antibody titers [35]. In this study, heterologous prime-boost administration was also the most efficient combination in terms of generating long-lived antibody responses; as shown by the induction of higher numbers of CSp-specific LLPCs upon restimulation with C-CSp. The effect of Ad35-CS/BCG-CS combination is of particular importance as LLPCs are thought to be instrumental for the acquisition of immunity against

clinical malaria in endemic areas [36]. Furthermore, a recent study has shown that a GMZ2 vaccine, a fusion protein consisting of the N-terminal portion of the glutamate rich protein (GLURP) fused to a C-terminal fragment of merozoite surface protein 3 (MSP3) plus the synthetic TLR4 agonist glucopyranosyl lipid A (GLA), elicits the highest number of LLPCs secreting cells specific for both the GMZ2 fusion protein and its two components [14].

In our current study, we tried to achieve simultaneous B- and T-cell responses against *P. falciparum* CSp. Heterologous prime-boost immunization regimens including vaccination of Ad35-CS followed by BCG expressing the *P. falciparum* CSp, could be one of the best approaches. The sporozoite challenge experiments are underway to define the protective efficacy of this prime-boost protocol.

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